JOURNAL OF BACTERIOLOGY, Aug. 1985, p. 464–471
0021-9193/85/080464-08$02.00/0

Vol. 163, No. 2

Structural Genes for Flagellar Hook-Associated Proteins in *Salmonella typhimurium*

MICHIO HOMMA,* KAZUHIRO KUTSUKAKE, AND TETSUO IINO

Laboratory of Genetics, Department of Biology, Faculty of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

Received 6 February 1985/Accepted 7 May 1985

The *flaW*, *flaU*, and *flaV* genes of *Salmonella typhimurium* LT2 were cloned into pBR322. These genes were mapped on the cloned DNA fragments by restriction endonuclease analysis and construction of the deletion derivatives. Their gene products were identified, by the minicell method, as proteins whose molecular weights were estimated to be 59,000 for the *flaW* product, 31,000 for the *flaU* product, and 48,000 for the *flaV* product. These values are identical to those of three species of hook-associated proteins (HAPs), namely, HAP1, HAP3, and HAP2. Furthermore, antibodies against HAP1, HAP3, and HAP2 specifically reacted with the gene products of *flaW*, *flaU*, and *flaV*, respectively. Therefore, we concluded that they are structural genes for HAPs. The antibodies against HAP1 and HAP3 also specifically reacted with the gene products of *flaS* and *flaT* of *Escherichia coli*, respectively. This indicates that these gene products are HAPs in *E. coli*. This result is consistent with the demonstration that *flaS* and *flaT* of *E. coli* are functionally homologous with *flaW* and *flaU* of *S. typhimurium*.

A bacterial flagellum consists of three distinct parts, i.e., a basal body, a hook, and a filament, and is inferred to be assembled in this sequence (1, 4, 5, 11, 16, 31). From genetic analysis of a large number of nonflagellate mutants, ca. 30 genes essential for flagellar formation have been identified in *Salmonella typhimurium* (14, 26, 35a). Among them, the mutants defective in *H1* and *H2*, *flaL*, *flaV*, *flaU*, or *flaW* have been shown to produce basal bodies with hooks but lacking filament portions (10, 32). This suggests that these genes play essential roles in filament formation, i.e., the final step of flagellar assembly. In fact, it has been shown that *H1* and *H2* are the structural genes for flagellin, which is the component protein of filaments (12). In mutants defective in *flaL*, active mRNA for flagellin was not detected (31). Thus, the *flaL* gene was inferred to be necessary for the transcription of flagellin genes. On the other hand, the *flaV*, *flaU*, or *flaW* mutants produce as much flagellin as do Fla$^+$ strains. Although the flagellin molecules produced by these mutants are able to polymerize in vitro at the same efficiency as those of Fla$^+$ strains, they cannot polymerize in vivo and are excreted into the culture medium (8). Therefore, these three genes are essential for in vivo polymerization of flagellin molecules at the tip of hooks (9, 10).

It had been believed that hooks were composed of a single kind of protein (19) which is the *flaFV* gene product (27). However, by analyzing hooks from the mutants defective in *H1* and *H2* or *flaL*, we showed that three species of minor proteins are present at the tip of hooks (9, 10). They were termed hook-associated proteins (HAPs), namely HAP1 (M_w = 59,000), HAP2 (M_w = 53,000 or 48,000), and HAP3 (M_w = 31,000). Furthermore, we showed that the hooks from the *flaV*, *flaU*, or *flaW* mutants lack one or more HAPs. The hooks from the *flaV* mutant contain HAP1 and HAP3 but lack HAP2; those from the *flaU* mutant contain only HAP1; and those from the *flaW* mutant contain a very small amount of HAP3 but lack HAP1 and HAP2. Thus, it has been inferred that *flaV*, *flaU*, and *flaW* control the presence of HAP2, HAP3, and HAP1 in hooks.

* Corresponding author.

From the above inference and related information (9), it is possible that *flaV*, *flaU*, and *flaW* are the structural genes for HAP2, HAP3, and HAP1, respectively. This work was carried out to confirm this assumption. We cloned the three genes with *Escherichia coli* as the host, because *E. coli* has advantages for the use of recombinant DNA techniques. Furthermore, almost all of the *fla* genes in *S. typhimurium* and *E. coli* are functionally homologous, and the chromosomal alignment is entirely the same in these two bacterial species (26, 35a). As a result, we obtained the DNA fragments harbored in plasmid pBR322 which restored motility to *flbC*, *flaS*, and *flaT* mutants of *E. coli* corresponding to *flaV*, *flaW*, and *flaU* mutants of *S. typhimurium*, respectively. Then we determined the locations of the *flaV*, *flaW*, and *flaU* genes of *S. typhimurium* on the cloned DNA fragments and identified their gene products as HAPs.

MATERIALS AND METHODS

Bacterial strains and plasmids. All of the bacterial strains used in this work are listed in Table 1. *E. coli* strains are derivatives of *E. coli* K-12. Plasmids used in this work are described in Table 1 and the text.

Media. L broth, L agar plates, and semisolid agar plates have been described previously (10). M9 salts contained (per liter of distilled water): Na_2HPO_4, 5.8 g; KH_2PO_4, 3.0 g; NaCl, 5.0 g; and NH_4Cl, 1.0 g. Antibiotics used and their respective concentrations were as follows: ampicillin, 30 μg/ml; and tetracycline, 10 μg/ml.

Manipulation of DNA. Conventional recombinant DNA techniques were performed as described by Maniatis et al. (29). The chromosomal DNA of *S. typhimurium* was prepared as described previously (26a).

Cloning of *fla* genes of *S. typhimurium*. The chromosomal DNA of *S. typhimurium*, digested by a restriction endonuclease, was ligated by T4 DNA ligase into pBR322 digested by the same endonuclease. The recombinant DNA was introduced into a *fla* mutant of *E. coli* EKK9 by the calcium chloride procedure (29), and then the cells were inoculated in lines on semisolid agar plates containing antibiotics. The *fla* mutant chosen either is defective in the *fla*

TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant genotype or comment	Reference
S. typhimurium LT2	Prototroph	15
E. coli		
YK102	F^- *his argE thyA thi rpsL uvrC mtl xyl pyrC46 gyrA*	24
YK405	F^- *araD139 lacU169 rpsL thi pyrC46 gyrA thyA*	23
YK410	F^- *araD139 lacU169 rpsL thi pyrC46 gyrA thyA his*	23
YK2047	*flaM* of YK102	27
YK4402	*flaZ* of YK410	20
YK4443	*flaS* of YK410	Komeda, unpublished data
YK4431	*flaT* of YK410	22
YK4104	*flaD* of YK410	33
YK4130	*hag* of YK410	21, 22
YK1101	*flbC* of YK410	21, 22
UH869	F^- *minA minB mgl(?) rpsL recA*	S. Harayama, unpublished data
TH912	F^- *minA minB rpsL*	7
KH802	F^- *met supE gal hsdR hsdM*$^+$	35
EKK9	F^- *thr leu met hsdR hsdM supE*	26a
MHE101	*flaM* of EKK9	This study
EKK11	*hag* of EKK9	26a
Plasmid		
pBR322	*bla*$^+$ *tet*$^+$	2
pLC24-46	ColE1 hybrid plasmid carrying the region I flagellar genes	23

gene which is to be cloned or is defective in a *fla* gene which is closely linked to the *fla* gene to be cloned. Transformed cells which formed swarms, i.e., spreading colonies, on the plate after incubation for 1 day were picked. After the transformed cells were grown in L broth, plasmid DNA was prepared from them by lysis with sodium dodecyl sulfate (SDS) (29) and reintroduced into the *fla* mutant by transformation. The antibiotic-resistant colonies were selected and tested for motility by sticking them into a semisolid agar plate. Motile clones thus obtained were kept and used for large scale preparation of plasmid DNA.

Isolation of *Bal* 31 deletion derivatives from pMH41. Plasmid pMH41 was digested by *Bam*HI. The digested sample was incubated at 60°C for 15 min and then digested by nuclease *Bal* 31 (Bethesda Research Laboratories, Rockville, Md.). The *Bal* 31-digested DNA and *Eco*RI linker (Takara, Kyoto, Japan) were mixed and treated with T4 DNA ligase. The ligated DNA was introduced into KH802 by transformation selecting for ampicillin resistance. Plasmid DNA prepared from the resulting transformants was digested by *Eco*RI and analyzed by agarose gel electrophoresis to estimate its molecular size.

Minicell method. The proteins programmed by a plasmid were analyzed by the minicell method as described by Reeve (30), with the following modifications. A 0.5-ml stationary-phase culture of a minicell-producing strain, UH869 or TH912, harboring the plasmid was inoculated into 250 ml of L broth with or without antibiotics. After 12 to 15 h of incubation at 37°C with vigorous shaking, the culture was centrifuged at 4,000 × *g* for 10 min to roughly remove viable cells. The resultant supernatant was recentrifuged at 22,000 × *g* for 20 min. The pellet was suspended in 1 ml of SG buffer (0.15 M NaCl, 4.2 mM Na_2HPO_4, 2.2 mM KH_2PO_4, 0.01% gelatin) and layered on top of sucrose gradient which was produced by freezing and thawing 32 ml of a solution of 20% (wt/vol) sucrose in SG buffer twice in a vertically positioned tube. The tube was centrifuged at 5,000 rpm for 15 min in a Beckman SW27 rotor. The minicell band (7 ml) was harvested with a syringe. After 10 ml of SG buffer was added to the minicell suspension, the minicells were collected by centrifugation at 27,000 × *g* for 10 min. They were suspended in 4 ml of SG buffer, and the absorbance of the suspension at 660 nm was measured. The minicells were collected again by centrifugation and suspended in methionine labeling buffer (2.6 g of methionine assay medium [Difco Laboratories, Detroit, Mich.] in 100 ml of M9 salts) to give an absorbance of 1 at 660 nm. This minicell suspension can be stored for at least 1 week at 0°C.

After 100 μl of the minicell suspension was incubated at 35°C for 5 min, 1 μl of [^{35}S]methionine (10 mCi/ml, 990 Ci/mmol, Amersham Corp., Arlington Heights, Ill.) was added, and the mixture was incubated for 30 min. The minicells were harvested by an Eppendorf centrifuge, washed, and suspended in 50 μl of TN buffer (50 mM Tris-hydrochloride [pH 7.8], 0.5% NaCl); 10 μl of TDG buffer (0.4 M Tris-hydrochloride [pH 6.8], 6% SDS, 38% glycerol, 0.004% bromophenol blue) and 5 μl of 2-mercaptoethanol were then mixed into the suspension. The mixture was heated at 100°C for 10 min and subjected to electrophoresis and fluorography.

Isolation of a protein specifically reacting with antibody from minicells. Minicells, which were radioactively labeled as above, were suspended in 100 μl of 1% SDS solution and heated at 100°C for 10 min. The SDS-solubilized suspension was diluted with 0.9 ml of TNET buffer (50 mM Tris-hydrochloride [pH 7.8], 0.15 M NaCl, 5 mM EDTA, 1% Triton X-100). The resultant suspension was centrifuged for 10 min in an Eppendorf centrifuge. The supernatant was mixed with 10 μl of trypsin inhibitor solution (1 mg/ml), 10 μl of antibody solution, and 10 mg of protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden). The mixture was incubated at 4°C for 12 h with shaking. The protein A-Sepharose was collected by the Eppendorf centrifuge and washed with 0.5 ml of TNET buffer. This washing procedure was repeated three times. The precipitate of protein A-Sepharose was suspended in 65 μl of the mixture (15 μl of 10% SDS, 35 μl of distilled water, 10 μl of TDG buffer, 5 μl of 2-mercaptoethanol). This suspension was heated at 100°C for 10 min and subjected to electrophoresis and fluorography.

Electrophoresis and fluorography. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (28).

After electrophoresis, an SDS-PAGE gel was shaken in methanol-acetic acid-water (5:1:4) for 1 h, and then fluorography was performed by the dimethyl sulfoxide–2,5-diphenyloxazole (DMSO-PPO) method of Bonner and Laskey (3) or the En^{3}Hance method of New England Nuclear Corp., Boston, Mass. The DMSO-PPO method was as follows. The gel was processed twice by 15-min soaks in DMSO with gentle shaking at 40°C, followed by a 30-min soak in 25% (wt/vol) solution of the scintillator PPO in DMSO with gentle shaking at 40°C. The gels were rehydrated in water for 1 h and dried thoroughly. The En^{3}Hance method was as follows. The gel processed by being soaked in En^{3}Hance, with gentle shaking for 1 h, was rehydrated in water and dried thoroughly. The dried gel was exposed to a Kodak X-Omat AR film at −70°C.

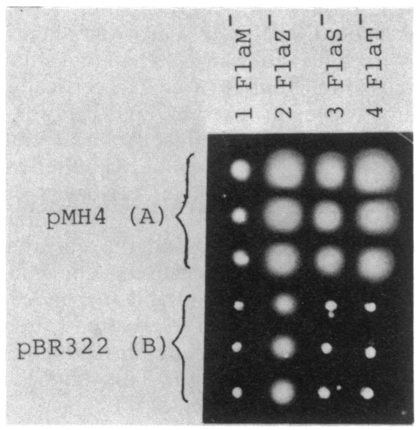

FIG. 1. Motility recovery of the *fla* mutants by pMH4. Plasmids pMH4 (A) and pBR322 (B) were introduced into the *fla* mutants as follows: lane 1, YK2047 (FlaM⁻); lane 2, YK4402 (FlaZ⁻); lane 3, YK4443 (FlaS⁻); and lane 4, YK4431 (FlaT⁻). The single colonies of ampicillin-resistant transformants were inoculated in a semisolid agar plate and incubated at 37°C for 10 h.

RESULTS

Cloning of the *flaW* and *flaU* genes of *S. typhimurium*. Chromosome DNA prepared from *S. typhimurium* LT2 was digested by *Eco*RI and ligated with a plasmid vector, pBR322, at the *Eco*RI site. A *flaM* mutant (MHE101) of *E. coli* was transformed by the resulting plasmids. The *flaM* gene is homologous with the *flaFIX* gene of *Salmonella* spp. Since the *flaFIX* gene is closely linked to the *flaW* and *flaU* genes (35a), we expected that the DNA fragment containing the *flaFIX* gene might also contain the *flaW* and *flaU* genes. One pBR322 clone with an additional *Eco*RI fragment which restored motilily to the *flaM* mutant was obtained. This plasmid contained an 11-kilobase (kb) *Eco*RI fragment and was named pMH4. In addition to the *flaM* mutant, pMH4 restored motility to the *flaZ*, *flaS*, or *flaT* mutants of *E. coli* (Fig. 1). These three genes are homologous with the *flaFX*, *flaW*, and *flaU* genes of *Salmonella* organisms. Therefore, we concluded that pMH4 at least contains the *flaFIX*, *flaFX*, *flaU*, and *flaW* genes of *S. typhimurium*.

A deletion derivative, pMH41, was constructed from pMH4 by digestion with *Bam*HI and ligation. It contained a 5.4-kb DNA fragment of *S. typhimurium* and restored motility to the *E. coli fla* mutants in the same way as did the original plasmid pMH4. We determined the cleavage sites of *Sal*I, *Mlu*I, and *Hpa*I on pMH41, and deletion derivatives were constructed from pMH41 by using *Sal*I or *Hpa*I (Fig. 2). A deletion derivative, pMH42 (Δ*Sal*I), restored motility to the *flaM* or *flaZ* mutants but not to the *flaS* or *flaT* mutants of *E. coli*. This indicates that the *Sal*I cleavage site of the cloned fragment on pMH42 is located either within the *flaW* gene or in a spacer region between the *flaFX* and *flaW* gene, since the order of the genes is *flaFIX-flaFX-flaW-flaU* (26, 35a). The other plasmid, pMH411 (Δ*Hpa*I), restored motility to only the *flaT* mutant of *E. coli*. Therefore, one of the *Hpa*I cleavage sites of *S. typhimurium* must be located either within the *flaFIX* gene or nearby, and the other one must be located either within the *flaW* gene or in a spacer region between the *flaU* and *flaW* genes.

Deletion derivatives were constructed from pMH41 by *Bal* 31 digestion and ligation with an *Eco*RI linker (Fig. 3). Among them, the #4r plasmid restored motility to the *flaS* mutant of *E. coli*, but the #7r plasmid with a larger deletion did not. These results indicate that one end of the *flaW* gene of *S. typhimurium* is located in the region which is present in the #4r plasmid but absent from the #7r plasmid. This end is inferred to be distal, since the direction of transcription is from *flaW* to *flaU* (K. Kutsukake, unpublished data). In the same way, it was shown that one end of the *flaFX* gene of *S. typhimurium* is located in the region which is present in the #36 plasmid but absent from the #22 plasmid. A further deletion derivative was constructed from the #3b plasmid by *Mlu*I digestion and ligation (Fig. 3). The resulting plasmid, #3bΔ*Mlu*I, restored motility to the *flaS* mutant but not to the *flaM* or *flaZ* mutants of *E. coli*. This shows that the *flaW* gene of *S. typhimurium* is located right outside of the *Mlu*I site.

Cloning of the *flaV* gene of *S. typhimurium*. Cloning of the *flaV* gene was carried out in a manner similar to that described in the foregoing section. One pBR322 clone with a *Bam*HI fragment which restored the motility to a *hag* mutant (EKK11) was obtained. Since the *hag* gene corresponds to the *H1* gene of *Salmonella* spp., which is closely linked to the *flaV* gene (35a), we expected that the DNA fragment containing the *H1* gene might also contain the *flaV* gene. This plasmid, pKK1301, contained a 12-kb *Bam*HI fragment. In addition to the *hag* mutant, pKK1301 restored motility to the *flbC* or *flaD* mutant of *E. coli*. These two genes cor-

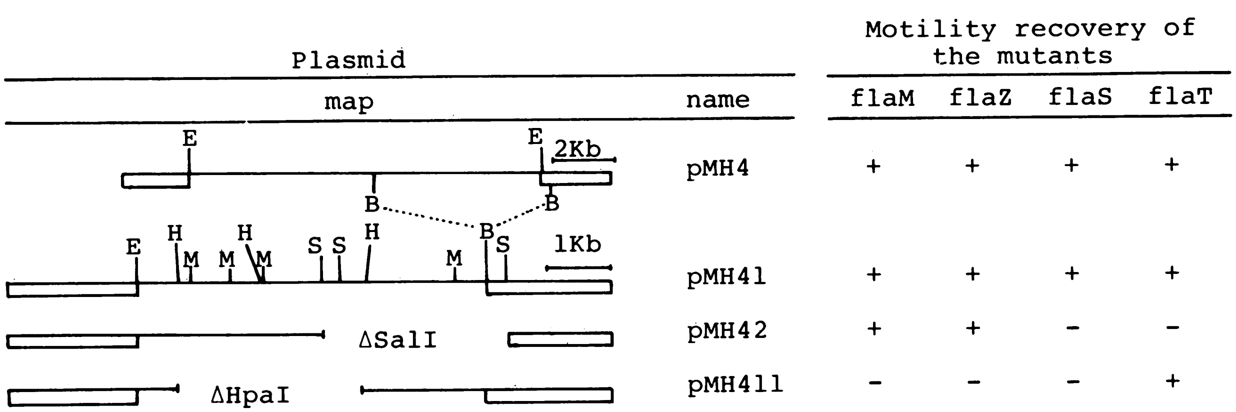

FIG. 2. Linearized physical map of plasmids carrying the *flaW* or *flaU* gene and motility recovery in the *fla* mutants. The motility recovery assay was carried out as described in the legend to Fig. 1. Symbols: single line, *S. typhimurium* DNA; and double line, pBR322 DNA. Restriction endonuclease sites are labeled as follows: B, *Bam*HI; E, *Eco*RI; H, *Hpa*I; M, *Mlu*I; and S, *Sal*I.

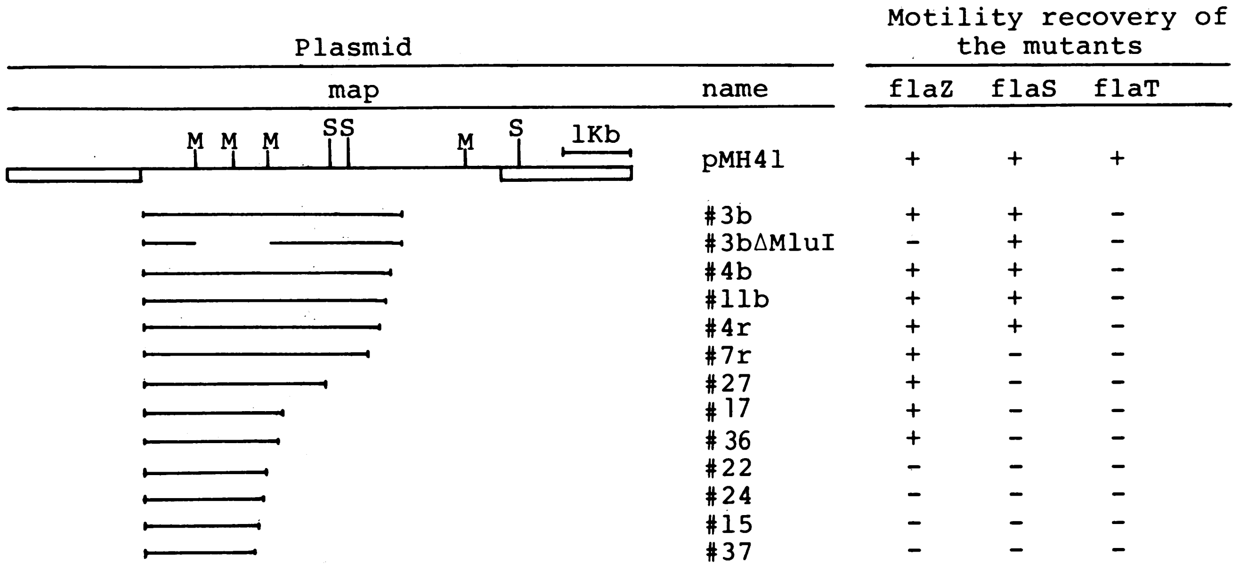

FIG. 3. *Bal* 31 deletion derivatives from pMH41 and motility recovery of the *fla* mutants. The motility recovery assay was carried out as described in the legend to Fig. 1. Symbols are the same as in the legend to Fig. 2.

respond to the *flaV* and *flaL* genes of *Salmonella* spp. Therefore, we can conclude that pKK1301 at least contains the *flaV*, *H1*, and *flaL* genes of *S. typhimurium*.

Plasmids pMH83 and pMH87 were obtained from pKK1301 by digestion with *Sal*I and ligation (Fig. 4). These two carried the same *Sal*I fragment of *S. typhimurium* but differed from each other in the orientation of the fragment. Both of them restored motility to the *flbC* or *hag* mutant but not to the *flaD* mutant of *E. coli*. The cleavage sites of *Eco*RI, *Mlu*I, *Hin*dIII, *Sac*II, and *Sal*I were mapped on the plasmids, and then deletion derivatives were constructed (Fig. 4). Plasmid pMH823 (Δ*Hin*dIII of pMH83) restored motility to the *flbC* and *hag* mutants of *E. coli*, but plasmid pMH872 (Δ*Eco*RI of pMH87) did not restore motility to either of the mutants. Plasmid pMH822 (Δ*Eco*RI of pMH83) recovered motility to the *hag* mutant only. Therefore, the 0.45-kb fragment of *Eco*RI in the cloned fragment is inferred to contain at least a part of the *flaV* gene of *S. typhimurium*, and one end of the *flaV* gene seems to be located between the *Eco*RI site of pMH872 and the *Hin*dIII site of pMH823. The *H1* gene of *S. typhimurium* has been cloned and also shown to be located in the 2.6-kb fragment between the *Sal*I site and the *Eco*RI site of pMH822, and the *H1* promoter is located at the region about 350 base pairs apart from the *Eco*RI site (Y.

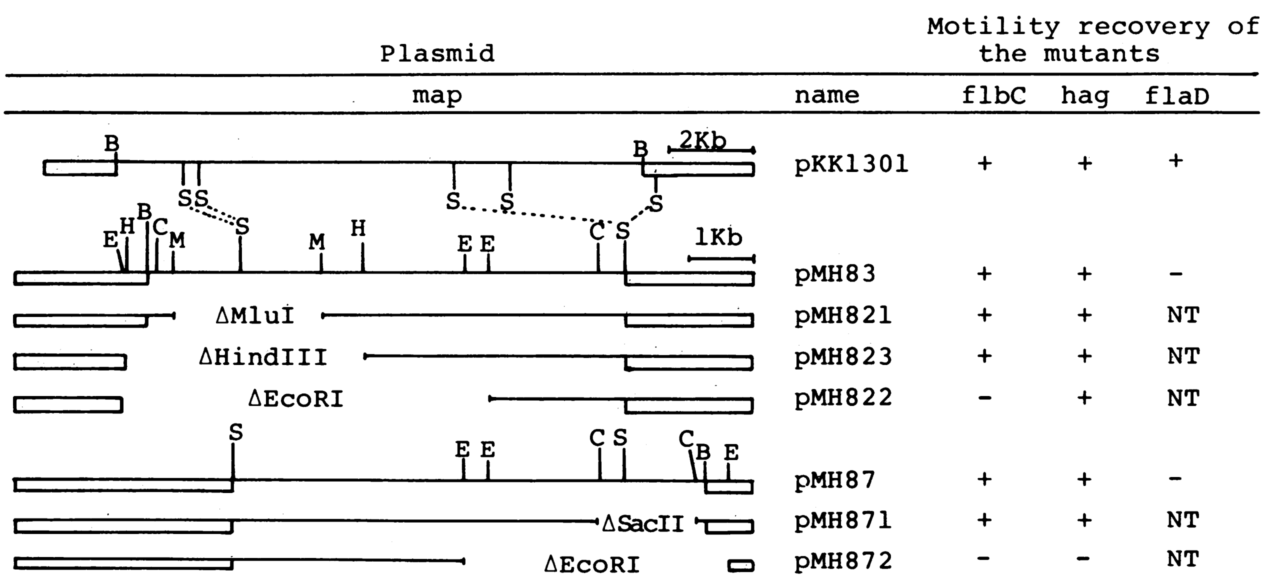

FIG. 4. Linearized physical map of plasmids carrying the *flaV* gene and motility recovery of the *fla* mutants. The plasmids were introduced into the *fla* mutants as follows: YK1101 (FlbC⁻); YK4130 (Hag⁻); and YK4104 (FlaD⁻). Motility recovery assay was carried out as described in the legend to Fig. 1. NT, Not tested. Symbols are the same as in the legend to Fig. 2, except for the following restriction endonuclease sites: H, *Hin*dIII; and C, *Sac*II.

Inoue, personal communication). Therefore, the other end of the *flaV* gene seems to be located between the *H1* promoter region and the *Eco*RI site of pMH872.

Identification of the *flaW*, *flaU*, and *flaV* gene products. Gene products programmed by the various plasmids constructed in the foregoing sections were analyzed by the minicell method. On the basis of the correspondence of genes inferred to exist in the plasmids to the appearance of the radioactively labeled proteins, the *flaU* gene was found to encode the 31,000-molecular-weight protein (e.g., the 31K protein) detected in pMH41 and pMH411 by SDS-PAGE (Fig. 5). Instead of the 31K protein, 15K protein was detected in the detection plasmid #3b.

By a similar method, the *flaW* gene was found to encode the 59K protein detected in plasmids pMH41, #3b, #4b, #4r, and #3bΔ*Mlu*I (Fig. 5). Instead of the 59K protein, 62K, 40K, and 31K proteins were detected in plasmids #7r, pMH42, and #27 respectively (Fig. 5).

By a similar method, the *flaV* gene was found to encode the 48K protein detected in plasmids pMH823, pMH821, and pMH871 (Fig. 6). The 52K protein, which was detected in plasmids pMH822, pMH823, pMH821, and pMH871, might be flagellin, namely the *H1* gene product, because its molecular weight has been estimated to be 52,000 by SDS-PAGE (25).

Reaction between the *flaW*, *flaU*, and *flaV* gene products and antibodies against HAPs. In a previous report (10), it was shown that *flaW*, *flaU*, and *flaV* control the presence of HAP1, HAP3, and HAP2, respectively. The molecular weights of HAP1, HAP3, and HAP2 were estimated to be 59,000, 31,000, and 48,000, respectively (10). These values are identical to those of the *flaW*, *flaU*, and *flaV* gene products which were shown in the foregoing section. These facts strongly support the conclusion that *flaW*, *flaU*, and *flaV* are the structural genes for HAP1, HAP3, and HAP2, respectively. To confirm this, we examined how the gene products of *flaW*, *flaU*, and *flaV* specifically reacted with antibodies against HAP1, HAP3, and HAP2, respectively (Fig. 7 and 8). As was expected, antibodies against HAP1 and HAP3 specifically reacted with the 59K and 31K proteins, respectively, among the radioactively labeled proteins programmed by pMH41, and antibody against HAP2 specifically reacted with the 48K protein among the radioactively labeled proteins programmed by pMH823.

The 15K protein programmed by plasmid #3b also specifically reacted with anti-HAP3 antibody, and the 62K, 40K, and 31K proteins programmed by plasmids #7r, pMH42, and #27, respectively, also specifically reacted with anti-HAP1 antibody (data not shown). Thus, these proteins may be abnormal products of the *flaU* and *flaW* genes caused by the deletions of parts of the plasmids.

A 48K protein was also detected in the radioactively labeled proteins programmed by pMH822, even though it did not have the *flaV* gene (Fig. 8). Its molecular weight was slightly different, and the protein did not react with antibody against HAP2 (data not shown). Therefore, the 48K protein of pMH822 was determined not to be the *flaV* gene product. The relative amounts of the *H1* and *flaV* gene products were considerably different among the plasmids used in Fig. 6. This may reflect the difference in DNA fragments of *S. typhimurium*, the influence of the location of the fragments in the vector, or both.

Correspondence between the *flaS* and *flaT* gene products of *E. coli* to the *flaW* and *flaU* gene products of *S. typhimurium*. The *flaW*, *flaU*, and *flaV* genes of *S. typhimurium* are homologous with the *flaS*, *flaT*, and *flbC* genes of *E. coli*, respectively (21, 26, 35a). Although the *flbC* gene product of *E. coli* has not been identified yet, the gene products of *flaS* and *flaT* of *E. coli* have been identified through specific

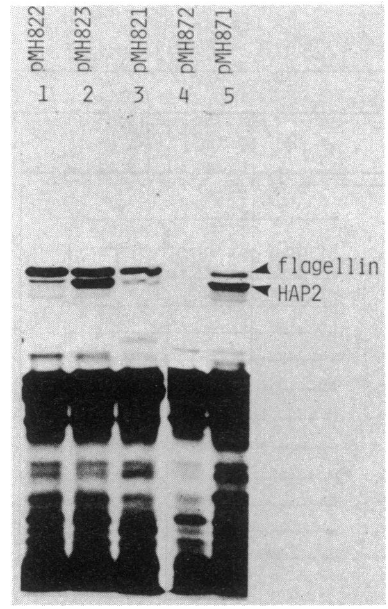

FIG. 6. Identification of the *flaV* gene product. Proteins were radioactively labeled in minicells containing plasmids as follows: lane 1, pMH822; lane 2, pMH823; lane 3, pMH821; lane 4, pMH872; and lane 5, pMH871. The proteins were analyzed by electrophoresis in a 10% polyacrylamide gel and fluorography.

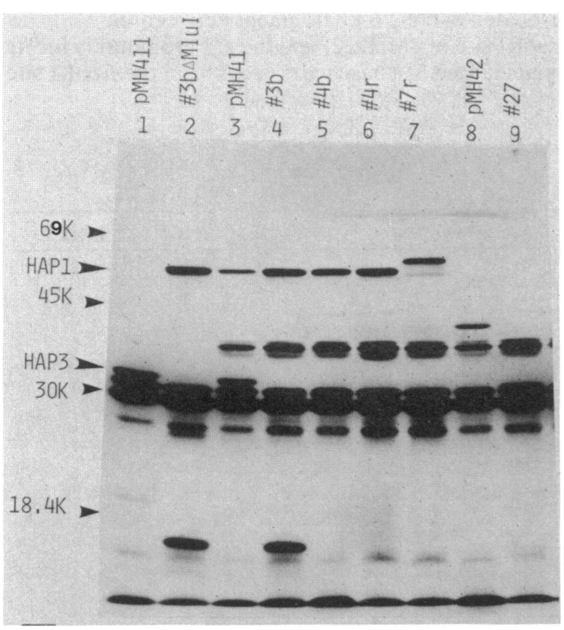

FIG. 5. Identification of the *flaW* or *flaU* gene product. Proteins were radioactively labeled in minicells containing plasmids as follows: lane 1, pMH411; lane 2, #3bΔ*Mlu*I; lane 3, pMH41; lane 4, #3b; lane 5, #4b; lane 6, #4r; lane 7, #7r; lane 8, pMH42; and lane 9, #27. The proteins were analyzed by electrophoresis in a 10% polyacrylamide gel and fluorography. Molecular weight markers are as follows: bovine serum albumin (69,000 [69K]), ovalbumin (45K), carbonic anhydrase (30K), and lactoglobulin (18.4K).

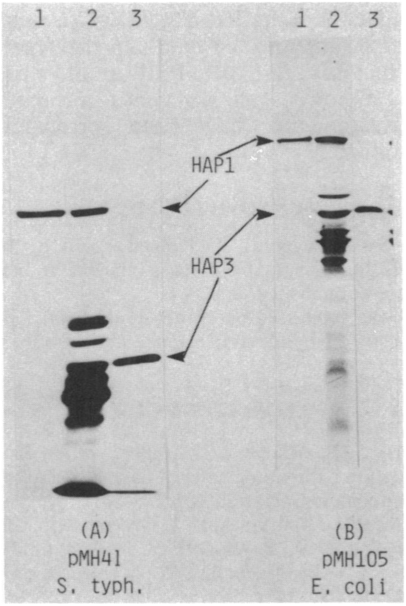

FIG. 7. Protein specifically reacting with antibody against HAP1 or HAP3. Proteins were radioactively labeled in minicells containing either the plasmid pMH41 carrying the *flaW* and *flaU* genes of *S. typhimurium* (A) or plasmid pMH105 carrying the *flaS* and *flaT* genes of *E. coli* (B). The proteins were incubated with anti-HAP1 antibody (lanes 1), not incubated with antibody (lanes 2), or incubated with anti-HAP3 antibody (lanes 3). The protein reacting with each antibody was isolated with protein A-Sepharose CL-4B. The proteins of (A) and (B) were analyzed by electrophoresis in 10 and 12% polyacrylamide gels, respectively, and fluorography.

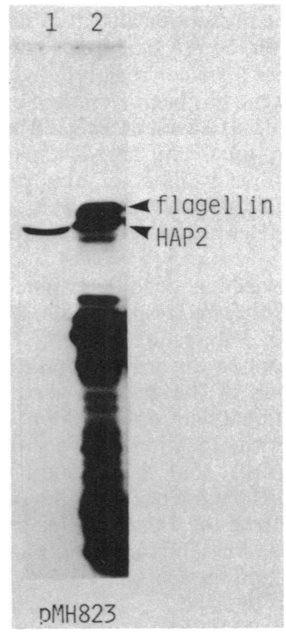

FIG. 8. Protein specifically reacting with anti-HAP2 antibody. Proteins were radioactively labeled in minicells containing the plasmid pMH823. They were either not incubated with antibody (lane 1) or incubated with anti-HAP2 antibody (lane 2). The protein reacting with the antibody was detected as described in the legend to Fig. 7A.

protein synthesis by lambda hybrid phages carrying these *fla* genes, and the molecular weights of their polypeptides, estimated by SDS-PAGE, were 60,000 for *flaS* and 35,000 for *flaT* (23).

We examined whether or not the gene products of *flaS* and *flaT* of *E. coli* reacted with antibodies against HAP1 and HAP3. The *Hin*dIII fragment (6.7 kb), which restored motility to the *flaZ*, *flaS*, or *flaT* mutant of *E. coli*, was subcloned into pBR322 from pLC24-46 which has been shown to carry these *fla* genes (23). Antibodies against HAP1 and HAP3 specifically reacted with 60K and 35K proteins, respectively, among the radioactively labeled proteins detected in the minicell carrying the subcloned plasmid pMH105 (Fig. 7). This result indicates that the 60K and the 35K proteins are HAPs in *E. coli*. On a 12% SDS-PAGE gel, the mobility of the *flaS* gene product of *E. coli* estimated as 60K protein was the same as that of the *flaW* gene product of *S. typhimurium* estimated as 59K protein (data not shown). Therefore, the difference between the estimated molecular weights of the corresponding gene products may be attributed to the difference in the SDS-PAGE conditions employed. On the contrary, the mobility of the *flaT* gene product of *E. coli* estimated as 35K protein was distinctly different from that of the *flaU* gene product of *S. typhimurium* estimated as 31K protein (data not shown).

DISCUSSION

The genes *H1* or *H2*, *flaL*, *flaV*, *flaU*, and *flaW* are essential for filament formation (10). Among them, the gene products of *flaW*, *flaU*, and *flaV*, which control the presence of HAP1, HAP3, and HAP2, respectively (10), were identified by cloning these genes (Fig. 5 and 6). Their molecular weights have been estimated by SDS-PAGE as 59,000 for the *flaW* product, 31,000 for the *flaU* product, and 48,000 for the *flaV* product, which are identical with those of HAP1, HAP3, and HAP2, respectively. Furthermore, we showed that these gene products specifically reacted with antibody against HAP1, HAP3, or HAP2 (Fig. 7 and 8); therefore, we can conclude that those genes are the structural genes for HAPs.

The locations of the HAP genes are illustrated on linearized physical maps from the following lines of evidence and inferences (Fig. 9). Taking the value of 110 as the average molecular weight of an amino acid, proteins with molecular weights of 59,000, 31,000, and 48,000 should be encoded in DNA fragments of 1.6, 0.85, and 1.3 kb, respectively. From the correspondence of the regions deleted in the derivative plasmids and complementation behavior of the mutants by the plasmids carrying these genes, the complete *fla* genes

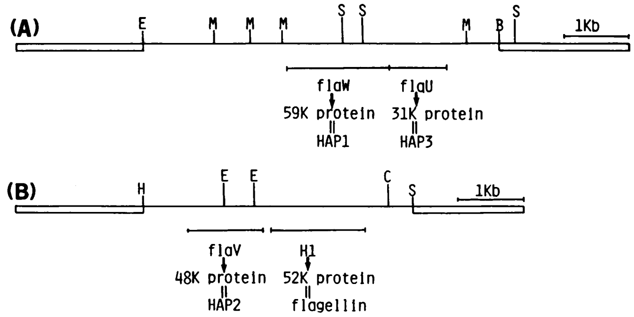

FIG. 9. Correspondence between the *flaW*, *flaU*, *flaV*, and *H1* genes and their products and locations on the physical maps of the plasmids pMH41 (A) and pMH823 (B). Symbols are the same as in the legend to Fig. 2.

were inferred to be present or absent in derivative plasmids (Fig. 2–4). Based on the physical maps of the plasmids (Fig. 9), we estimate that a spacer region between *flaW* and *flaU* is very small. No gene has been identified between them, and they are closely linked to each other. *flaW* and *flaU* belong to the same transcriptional unit (K. Kutsukake, unpublished data). The molecular weights of abnormal proteins programmed by the deletion derivatives (Fig. 5) conform to the deletions of the genes estimated from the locations of the genes in Fig. 9.

The DNA sequence has been determined in the promoter region of *H1* in *Salmonella* spp. or the homologous gene, *hag*, in *E. coli* (34). The results indicated that another gene, *rfs*, may be transcribed toward the opposite direction from the promoter region of the flagellin gene *H1* or *hag*. This gene might be identical with *flaV* of *Salmonella* spp. or *flbC* of *E. coli*, because the location is adjacent to *H1* or *hag* on the same side as *rfs* (21, 35a). A part of the *flaV* gene must be located in the *Eco*RI fragment of 0.45 kb, which is placed at ca. 0.35 kb apart from the *H1* promoter region on pMH823 (Fig. 9). Therefore, we can regard the N-terminal region of *rfs* as that of *flaV*, and the location of the *flaV* gene is shown on the map of Fig. 9.

Two species of HAP2 have been identified by different molecular weight, 53,000 or 48,000 (9, 10). HAP2 with a molecular weight of 53,000 (53K HAP2) was identified from the study of the strains which seem to have the *flaV* gene from *Salmonella* sp. strain SJ925 with the gt-type flagellin gene, and 48K HAP2 was identified from the strains which have the *flaV* gene from *S. typhimurium* LT2 with the i-type flagellin gene. We cloned the *flaV* gene from *S. typhimurium* LT2 in this study. The gene product with a molecular weight of 48,000 specifically reacted with anti-HAP2 antibody (Fig. 8), although the antibody had been prepared from 53K HAP2 (9). On the other hand, it has been shown by immunoelectron microscopic observation that the antibody is bound to the top of the hook structure which is composed of 53K HAP2, but it is not bound specifically to the top of that which is composed of 48K HAP2 (9). This means that the antigenic specificities of the structure surface are different between 53K HAP2 and 48K HAP2, which still hold the common antigenic site concealed in the structure. The common antigenic site may reflect a functional domain of HAP2.

The gene products of *flaS* and *flaT* of *E. coli*, corresponding to *flaW* and *flaU* of *Salmonella* spp., also reacted with antibodies against *Salmonella* HAP1 and HAP3, respectively (Fig. 7). This means that *flaS* and *flaT* of *E. coli* are also the structural genes for HAPs, and they have common antigenicity in these two species. The antigenicity of flagellar filaments is very heterogenous among bacterial species (12). However, the antigenicity of flagellar hooks is less heterogenous than that of filaments (17, 18). Compared with the hooks, filaments are the target for strong selection pressure in immune system of animals, because filament length is ca. 10 μm (13), but hook length is no more than 50 nm (10). Moreover, bacterial motility is inhibited by antifilament serum but not by antihook serum (10). The fact that antibodies against HAP1 and HAP3 of *Salmonella* spp. reacted with those of *E. coli* may suggest that the antigenicities of HAP1 and HAP3 are not so heterogenous among bacterial species.

The final step of flagellar assembly, i.e., filament formation, requires the *H1* or *H2*, *flaL*, *flaV*, *flaU*, and *flaW* genes (10). *H1* and *H2* have been shown to be the structural genes for flagellin (12). It has been inferred that *flaL* is the gene for a positive regulator of the *H1*, *H2*, *flaV*, *flaU*, and *flaW* genes (K. Kutsukake, unpublished data). In the present study, it was shown that *flaV*, *flaU*, and *flaW* are the structural genes for HAPs. Therefore, all six genes responsible for the filament formation step have been correlated with their specific functions.

ACKNOWLEDGMENTS

We thank S. Yamaguchi, Y. Komeda, and S. Harayama for bacterial strains and invaluable discussions and Y. Inoue for allowing us to quote unpublished data.

This work was supported by grants in aid for scientific research from the Ministry of Education, Science and Culture in Japan.

LITERATURE CITED

1. **Abram, D., J. R. Mitchen, H. Koffler, and A. E. Vatter.** 1970. Differentiation within the bacterial flagellum and isolation of the proximal hook. J. Bacteriol. **101**:250–261.
2. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles. II. A multiple purpose cloning system. Gene **2**:95–113.
3. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
4. **DePamphilis, M. L., and J. Adler.** 1971. Fine structure and isolation of the hook-basal body complex of flagella from *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol. **105**:384–395.
5. **Dimmitt, K., and M. I. Simon.** 1971. Purification and partial characterization of *Bacillus subtilis* flagellar hooks. J. Bacteriol. **108**:282–286.
6. **Doetsch, R. N., and R. D. Sjoblad.** 1980. Flagellar structure and function in eubacteria. Annu. Rev. Microbiol. **34**:69–108.
7. **Harayama, S., T. Oguchi, and T. Iino.** 1984. Does Tn10 transpose via the cointegrate molecule? Mol. Gen. Genet. **194**:444–450.
8. **Homma, M., H. Fujita, S. Yamaguchi, and T. Iino.** 1984. Excretion of unassembled flagellin by *Salmonella typhimurium* mutants deficient in hook-associated proteins. J. Bacteriol. **159**:1056–1059.
9. **Homma, M., and T. Iino.** 1985. Locations of hook-associated proteins in flagellar structure of *Salmonella typhimurium*. J. Bacteriol. **162**:183–189.
10. **Homma, M., K. Kutsukake, T. Iino, and S. Yamaguchi.** 1984. Hook-associated proteins essential for flagellar filament formation in *Salmonella typhimurium*. J. Bacteriol. **157**:100–108.
11. **Holt, S. C., and E. Canale-Parola.** 1968. Fine structure of *Spirochaeta stenostrepta*, a free-living, anaerobic spirochete. J. Bacteriol. **96**:822–835.
12. **Iino, T.** 1969. Genetics and chemistry of bacterial flagella. Bacteriol. Rev. **33**:454–475.
13. **Iino, T.** 1974. Assembly of *Salmonella* flagellin *in vitro* and *in vivo*. J. Supramol. Struct. **2**:372–384.
14. **Iino, T.** 1977. Genetics of structure and function of bacterial flagella. Annu. Rev. Genet. **11**:161–182.
15. **Iino, T., and M. Enomoto.** 1966. Genetical studies of non-flagellate mutants of *Salmonella*. J. Gen. Microbiol. **43**:315–327.
16. **Johnson, R. C., M. P. Walsh, B. Ely, and L. Shapiro.** 1979. Flagellar hook and basal complex of *Caulobacter crescentus*. J. Bacteriol. **138**:984–989.
17. **Kagawa, H., S.-I. Aizawa, S. Yamaguchi, and J.-I. Ishizu.** 1979. Isolation and characterization of bacterial flagellar hook proteins form salmonellae and *Escherichia coli*. J. Bacteriol. **138**:235–240.
18. **Kagawa, H., S. Asakura, and T. Iino.** 1973. Serological study of bacterial flagellar hooks. J. Bacteriol. **113**:1474–1481.
19. **Kagawa, H., K. Owaribe, S. Asakura, and N. Takahashi.** 1976. Flagellar hook protein from *Salmonella* SJ25. J. Bacteriol. **125**:68–73.
20. **Komeda, Y.** 1982. Fusions of flagellar operons to lactose genes

on a Mu *lac* bacteriophage. J. Bacteriol. **150**:16–26.

21. **Komeda, Y., K. Kutsukake, and T. Iino.** 1980. Definition of additional flagellar genes in *Escherichia coli* K-12. Genetics **94**:277–290.
22. **Komeda, Y., N. Ono, and H. Kagawa.** 1984. Synthesis of flagellin and hook subunit protein in flagellar mutants of *Escherichia coli* K-12. Mol. Gen. Genet. **194**:49–51.
23. **Komeda, Y., M. Silverman, P. Matsumura, and M. Simon.** 1978. Genes for the hook-basal body proteins of the flagellar apparatus in *Escherichia coli*. J. Bacteriol. **134**:655–667.
24. **Komeda, Y., M. Silverman, and M. Simon.** 1977. Genetic analysis of *Escherichia coli* K-12 region I flagellar mutants. J. Bacteriol. **131**:801–808.
25. **Kondoh, H., and H. Hotani.** 1974. Flagellin from *E. coli* K-12: polymerization and molecular weight in comparison with *Salmonella* flagellins. Biochim. Biophys. Acta **336**:119–126.
26. **Kutsukake, K., T. Iino, Y. Komeda, and S. Yamaguchi.** 1980. Functional homology of *fla* genes between *Salmonella typhimurium* and *Escherichia coli*. Mol. Gen. Genet. **178**:59–67.

26a. **Kutsukake, K., T. Nakao, and T. Iino.** 1985. A gene for DNA invertase and an invertible DNA in *Escherichia coli* K-12. Gene **34**:343–350.

27. **Kutsukake, K., T. Suzuki, S. Yamaguchi, and T. Iino.** 1979. Role of gene *flaFV* on flagellar hook formation in *Salmonella typhimurium*. J. Bacteriol. **140**:267–275.
28. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
29. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Reeve, J.** 1979. Use of minicells for bacteriophage-directed polypeptide synthesis. Methods Enzymol. **68**:493–503.
31. **Suzuki, H., and T. Iino.** 1975. Absence of messenger ribonucleic acid specific for flagellin in non-flagellate mutants of *Salmonella*. J. Mol. Biol. **95**:549–556.
32. **Suzuki, T., T. Iino, T. Horiguchi, and S. Yamaguchi.** 1978. Incomplete flagellar structures in nonflagellate mutants of *Salmonella typhimurium*. J. Bacteriol. **133**:904–915.
33. **Suzuki, T., and Y. Komeda.** 1981. Incomplete flagellar structures in *Escherichia coli* mutants. J. Bacteriol. **145**:1036–1041.
34. **Szekely, E., and M. Simon.** 1983. DNA sequence adjacent to flagellar genes and evolution of flagellar-phase variation. J. Bacteriol. **155**:74–81.
35. **Velten, J., K. Fukada, and J. Abelson.** 1976. *In vitro* construction of bacteriophage λ and plasmid DNA molecules containing DNA fragment from bacteriophage T4. Gene **1**:93–106.

35a. **Yamaguchi, S., H. Fujita, T. Tatra, K. Kutsukake, M. Homma, and T. Iino.** 1985. Genetic analysis of three additional *fla* genes in *Salmonella typhimurium*. J. Gen. Microbiol. **130**:3339–3342.